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(54) Title: DETERMINATION OF POLYPEPTIDES AND PROTEINS IN SOLUTIONS AND BIOLOGICAL FLUIDS

(57) Abstract

A method for the qualitative and quantitative determination of a polypeptide or protein analyte present in a biological fluid or a solution is provided, utilizing a competitive-type assay system in which the competitive reagent is a labelled peptide fragment present in the amino acid sequence of the analyte. This method is exemplified by the determination of human nerve growth factor. Also provided are a kit for the determination of polypeptide or protein analytes employing this assay method, and a method useful in human and veterinary medicine for the diagnosis and/or monitoring of a variety of diseases and therapies therefor in which a characteristic polypeptide or protein provides an effective diagnostic tool.

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Determination of polypeptides and proteins in solutions and biological fluids

The present invention relates to analytical methods for qualitatively and quantitatively determining the presence of polypeptides and proteins in solutions and biological fluids. In particular, it relates to a competitive-type analytical assay system employing antibodies specific for a portion of the amino acid sequence of a polypeptide or protein, and the polypeptide, protein, or a portion thereof as an analytical reagent.

Analytical techniques for molecules of biological importance must be sensitive and specific. Many techniques for analyte assay in biological fluids have been developed to date (Levi Montalcini R. et al., J. Exp. Zool. 116:321, 1951; Varon S. et al., Biochemistry 6:2202, 1967; Angeletti R. H., Proc. Natl. Acad. Sci. USA 65:668, 1970; Harper G. P. et al., Nature 279:160, 1979; Goldstein L. D. et al., Neurochem. Res. 3:175, 1978; Walker P. et al., Life Science 26:195, 1980; Calissano P. et al., Hormonal Prot. Peptides, XII:2, 1984; Italian Patent Application No. 47745A88; Callegaro et al., Proceedings of the Satellite to the 20th Annual Meeting of the American Society for Neurochemistry, "Trophic Factors and the Nervous System", Columbus, Ohio, March 12-14, 1989, Raven Press, Fidia Research Series - in press; Kingsbury D.T., Falkow S., "Rapid Detection and Identification of Infectious Agents", Academic Press; Levi Montalcini R., Science 1237, 1154, 1987).

All th se methodologies rely on the availability of one or more monoclonal and/or polyclonal antibodies, or on the availability of the analyte which activates the detection system. In all cases, fairly large quantities of analyte were, and still are, necessary to obtain antibodies by immunization and cloning methodologies, or to develop labelling systems.

The presence or concentration of antigenic substances in biological fluids can be determined by immunoassay techniques. Such techniques are based on the formation of a complex between the antigenic substance being assayed and an antibody in which a member of the complex may be labelled to permit detection or quantitative analysis after separation of the complexed labelled antigen or antibody from uncomplexed labelled antigen or antibody.

In the competitive-type immunoassay technique, the antigenic substance in a sample test fluid competes with a known quantity of labelled antigen for a limited quantity of antibody binding sites. Consequently, the amount of labelled antigen bound to the antibody is inversely proportional to the amount of antigen in the sample. In contrast, immunometric assays employ a labelled antibody, and the amount of labelled antibody associated with the complex is directly proportional to the amount of antigenic substance in the sample fluid.

One currently employed method for identifying the biologically important regions of a protein molecule involves the determination of its hydrophobic and hydrophilic regions. Since hydrophilic regions are normally exposed on the surface of the protein molecule, they are likely to exercise a particular biological function. It may be expected, for example, that they could act as receptor binding regions, or that they represent antigenic sites, capable of inducing the formation of specific antibodies.

Present technology has made it possible to identify different hydrophilic and hydrophobic sites in polypeptides

and prot ins corresponding to regions of different antigenicity. For example, Hopp and Woods ((1961) Proc. Natl. Acad. Sci. USA 78:3824) developed an algorithm which is capable of producing the hydrophilic profile of a protein molecule, thus making it possible to assess whether a given peptide of the polypeptide chain tends to place itself on the surface or in the interior of the protein structure. This method takes into consideration a peptide chain between 6 and 10 amino acid residues long, calculating its hydrophilic value by the coefficients assigned to the single amino acid residues. By shifting section by section along the sequence one amino acid at a time, a complete diagram can be obtained (Meier R. et al., EMBO J. 5:1489, 1986). The most hydrophilic sequences correspond to the positive peaks of the Hopp and Woods diagram, and single out the zones most exposed to the solvent. These portions of the molecule could therefore represent potential antigenic sites, and also active sites, i.e., exposed regions of the molecule capable of interacting with other biological macromolecules.

Current technology has evolved to the point where sensitive, highly specific analytical methods must be devised to facilitate polypeptide and protein assay, including polypeptides and proteins with as yet incompletely known biological activities. One such polypeptide is the nerve growth factor (NGF).

There have been numerous in vitro and in vivo studies of NGF on animal models. These have advanced to the state where a potential use for NGF can be hypothesized in the treatment of Alzheimer's disease (Science 243:11, 1989). Recently, other important hypotheses have been put forward regarding a mediatory role for NGF between the central nervous system and the immune system (Aloe L. et al., Proc. Natl. Acad. Sci. USA 1983, 6184:1986; Spillantini M.G. et al., Proc. Natl. Acad. Sci. USA 1986, 8555:1989). There is

th r for an urgent n d for a quantitative analytical technique which will facilitate the determination of human NGF, and other medically important polypeptides and proteins, in biological fluids or culture media. Consequently, an analytical method and suitable, specific reagents for the molecule to be analyzed are also needed.

Among the available immunological analytical methods for proteins, the "competitive" system is certainly the most sensitive in terms of specificity. Determination of an analyte in biological liquids by a competitive-type reaction system is performed by incubating the sample, with or without analyte, with a support to which anti-analyte antibodies are bound, usually defined as the solid phase. Subsequently, when the analyte which has not reacted with the antibodies has been eliminated, the solid phase is washed with suitable buffers and incubated with the analyt in question, which can be directly or indirectly labelled with a visualizing agent, for example with a biotin-avidin-enzyme system (Johnson B. et al., J. Immunol. Meth. 115: 219, 1988). There are many detection methods presently available which involve visualization of the interaction between analyte and antibody in the competitive system; many supports, to which antibodies can be adsorbed, are also available. The supports and detection systems ar constantly being modified, but the fundamental usefulness of a quantitative and qualitative analytical assay based on a competitive system lies in its specificity, i.e., in its ability to detect unequivocally and exclusively the presence of the analyte of interest in a liquid. Clearly, the ability to recognize the analyte exclusively in one particular structural form, such as that associable with its biological activity, increases the specificity of the analytical assay and consequently the biological significance of the quantitative detection of the analyte in question. In analytical assays, competitive-type specificity is mainly achieved by anti-analyte antibodies and by the labelled analyte used as a tracer.

Accordingly, it is an aspect of th present invention to provide a method for the qualitative or quantitative determination of a polypeptide or protein analyte present in a biological fluid or solution, comprising the steps of:

(a) producing antibodies to a peptide fragment of said polypeptide or protein;

(b) reacting a sample of said biological fluid or solution with said antibodies in the presence of said peptide fragment labelled so as to allow detection of complexes of said antibodies with said labelled peptide fragment;

(c) removing any unreacted biological fluid or solution and labelled peptide fragments from the reaction mixture of step (b);

(d) incubating the remaining antibodies with a reagent capable of detecting antibody-labelled peptid fragment complexes; and

(e) qualitatively and quantitatively detecting or measuring said antibody complexes of step (d).

It is another aspect of the present invention to provide a method for the diagnosis and/or monitoring of a disease, or a therapy therefor, such as a neuroimmunoendocrine disease, a neuroimmunodegenerative disease, a disease of the central nervous system, or a disease of the peripheral nervous system in a human or animal, comprising determining the level of a diagnostically relevant polypeptide or protein in a biological fluid of said human or animal according to the method provided above.

It is a further aspect of the present invention to provide a kit for the qualitative or quantitativ determination of a polypeptide or protein analyte present in a biological fluid or solution, comprising:

anti-analyte antibodies specific for a peptide fragment of said polypeptide or protein analyt which are capable of r cognizing said polypeptide or protein analyte; and

a peptide fragment which is capable of being recognized by said antibodies, and which is also capable of being detected by a detection system.

It is yet a further aspect of the present invention to provide a method for the determination of human nerve growth factor in a biological fluid or solution, comprising the steps of:

producing polyclonal antibodies to said human nerve growth factor by immunizing a rabbit with synthetic peptide 20-36 of human nerve growth factor;

adsorbing said polyclonal antibodies onto the surface of the wells of a polycarbonate microtitration plate;

placing samples of said biological fluid or solution in said wells in the presence of peptide 20-36 conjugated to biotin and incubating for 24 hours at room temperature;

removing any excess, unreacted reagent and washing said wells with buffer containing bovine albumin and a detergent;

adding avidin-conjugated enzyme to said wells and incubating for a period of time effective to allow avidin-biotin complexes to form;

removing excess reagent by washing said wells;

adding enzyme substrate to said washed wells in an appropriate buffer and incubating so as to allow formation of the enzymatic reaction end-product;

measuring the amount of said end-product formed; and

relating the amount of said end-product formed to the amount of said human nerve growth factor with the aid of a standard curve for human nerve growth factor.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawings provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments

of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed descriptions taken in conjunction with the accompanying drawings, all of which are given by way of illustration only, and are not limitative of the present invention, in which:

Figure 1 shows the reactivity of human NGF anti-peptide 20-36 polyclonal antibodies compared to that of polyclonal antibodies produced by immunization with the 2.5S form of NGF against human NGF in a Western blot: a) polyclonal antibodies produced in rabbit by immunization with NGF peptide 20-36; b) polyclonal antibodies produced in rabbit by immunization with the 2.5S form of NGF and purified by affinity chromatography on peptide 20-36 immobilized on a solid support and on immobilized NGF, respectively. The presence of polyclonal antibodies was detected by means of a commercially available amplification system.

Antisera which are not NGF-related do not react with this protein in Western blots (data not shown).

Detection system: blotted proteins were visualized with anti-rabbit AuroProbe BL plus secondary antibodies (Janssen) following the manufacturer's instructions.

Molecular weights: the Pharmacia low-molecular-weight marker calibration kit was employed.

Figure 2 is a standard curve showing absorbance readings at 492 nm for samples treated as described in Example 1, infra, disclosing a linear correspondence between the analyte, human NGF, and optical density in the presence competitive immunological assay system.

X axis = ng/ml human NGF

Y axis = B/Bo where:

Bo = absorbance value (in nm) of the standard concentration of the tracer (peptide 20-36 labelled with biotin);

B = absorbance value obtained in the presence of the relative quantity of competitor (human NGF).

The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description of the invention should not be construed to unduly limit the present invention, as modifications and variations in the embodiments herein discussed may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references discussed in the present specification are herein incorporated by reference in their entirety.

The purpose of the present invention is to provide an assay for a soluble polypeptide or protein or their fragments based on the calculation of the hydropathic profile derived from its gene sequence and the consequent amino acid sequence.

The invention is illustrated hereafter by Examples 1 and 2, and exemplifies the assay of the biologically active form of human NGF obtained by recombinant DNA methodology (Italian patent application No. 48564A89). Moreover, within the context of the present invention, the use of NGF must be considered illustrative and not limiting in any way, since the experimental approach on which the invention is based can be applied to all polypeptides and proteins.

NGF from mouse glands behaves as a 7S-type protein complex (molecular w ight about 140,000 Daltons) formed by thre different subunits (α,β,γ) coordinating a Zn^{+} atom.

The most interesting part of the 7S molecule, with regard to its biological activity, consists of two polypeptide chains, each with a molecular weight of 13,250 Daltons and formed by 118 amino acids. Each chain or monomer has three disulfide bridges, which form covalent bonds between two cysteine residues, and which confer great stability to the three-dimensional structure of the protein. The two NGF monomers, joined together by weak bonds, form a dimer with a molecular weight of 26,500 D. It has been demonstrated that the biological activity is associated with the dimer called 2.5S, or more commonly, βNGF.

Among the possible hydrophilic regions of NGF in the Hopp and Woods diagram, the amino acid portion identifiable as 20-36, i.e., the amino acids at positions 20-36 of the NGF polypeptide, was chosen to illustrate the utility of the present invention. The synthetic peptide 20-36 of NGF can be used to produce polyclonal antibodies capable of selectively recognizing and interacting not only with the peptide itself, but also with the entire NGF molecule. This fragment is common to a variety of animal species, facilitating analysis of the NGF protein therein.

The anti-NGF antibodies used in the solid phase, infra, i.e., adsorbed onto the support, were produced by immunizing rabbits via multiple site intradermal injections of the free peptide, absent a carrier, in complete Freund's adjuvant, with a specific NGF fragment, a peptide comprising the amino acid sequence corresponding to portion 20-36. These were purified by affinity chromatography on the same peptide, immobilized on a solid support, obtained from a different synthetic preparation from the one used for immunization. These anti-peptide 20-36 polyclonal antibodies were assessed for cross-reactivity with human NGF in its biologically active form, 2.5S or βNGF, cloned and expressed in eukaryotic cells (Italian patent application No. 48564A89).

Assessment of the reactivity of these NGF anti-peptide 20-36 polyclonal antibodies is shown in Figure 1, where it can be seen that rabbit polyclonal antibodies produced by immunization with either NGF peptide 20-36 or the 2.5S form of NGF exhibit the same reactivity with human NGF upon Western blotting.

EXAMPLE 1

Determination of human NGF in culture medium in polycarbonate microtitration plates.

A. Methods

A.1. Coating of the microtitration plate wells with antibodies.

The wells in the polycarbonate microtitration plates were adsorbed on the inside with the previously characterized anti-peptide 20-36 polyclonal antibodies of the NGF polypeptide, diluted in a bicarbonate buffer at pH 9.0, by incubation for 24 hours at room temperature.

The concentration of antibody used to adsorb to the wells ranged between 0.1-10 µg/ml, preferably 1 µg/ml, in carbonate buffer, 0.1M, pH 8.5. Wells were coated with 20 µl-100 µl, preferably 50 µl, of this solution. The value of 50 µl corresponds to 50 ng/well, indicated in Fig. 2.

The wells were then washed with 0.15 mol./litre of phosphate buffer, pH 7.2.

A.2. Incubation of the microtitration plates with the sample to be analyzed.

50 µl of analyte sample, at different concentrations, were placed in the wells of the microtitration plate in the presence of peptide 20-36 labelled with biotin (hapten-p ptide), and left to incubate for 24 h urs at room temperature.

As indicated in Figure 2, the concentration of NGF ranged between 0.1-100 ng/ml. The buffer employed was PBS,

containing 0.1% bovine serum albumin. Th quantity of labelled peptide 20-36 ranged from 2-20 ng/ml, preferably 10 ng/ml. This corresponded to 0.5 ng/well.

Any excess, unreacted reagent was then removed, and the wells of the microtitration plate were then washed with 0.15 mol./litre of phosphate buffer, pH 7.2, containing 2% bovine albumin and a non-ionic detergent such as 0.05% Tween 20.

A.3. Incubation with avidin-labelled enzyme.

The wells prepared as described in Section A.2. were filled with 50 µl of a commercially obtained tracer composed of avidin-labelled enzyme, such as horseradish peroxidase diluted in 0.15M phosphate buffer, pH 7.2, containing 0.05 % Tween 20 and 2% bovine albumin. This was incubated for 4 to 6 hours at room temperature or at 37°C. Any excess reagent was removed by washing with 0.15M phosphate buffer, pH 7.2.

A.4. Incubation with the enzyme substrate.

The wells treated as described in Section A.3. were filled with 50 µl of a mixture of 0.4 mg/ml of orthophenylenediamine and 0.2 mg/ml of hydrogen peroxide in 0.1M citrate buffer at pH 5.0. After incubation for 15 minutes, 50 µl of sulfuric acid, 2.5 mol./litre, were added to the wells of the microtitration plates.

A.5. Absorbance measurement.

The absorbance of the solutions in the wells of the microtitration plates was measured spectrophotometrically at a wavelength of 492 nm (OD 492 nm).

B. Results.

Absorbance readings, taken as described above for the wells containing solutions of human NGF at different concentrations in the culture medium, are plotted in Figure

2, where it can be se n that there is obtained a linear relationship between OD 492 nm and increasing concentrations in ng/ml of human NGF in the analyte sample. Such a standard curve permits the determination in a quantitative manner of human NGF in a sample of interest to be analyzed for the presence of this protein.

EXAMPLE 2

Test kit for the determination of human NGF.

The present invention also concerns a kit containing components necessary to detect the protein or polypeptide of interest. This kit must at least contain anti-peptide antibodies unbound or bound to a support, and the analyte polypeptide or protein, or one of their specific segments, bound to a labelling agent.

Human NGF was determined as described in Example 1 using a multi-assay kit. This kit contained the following:

- Polycarbonate microtitration plates with wells in which are adsorbed anti-peptide 20-36 polyclonal antibodies which recognize NGF in its form known as 2.5S or βNGF, and its non-dimeric forms;

- vials containing specific tracers comprising amino acid sequences recognized by the anti-peptide 20-36 antibody;

- bottles of phosphate buffer, pH 7.2, containing bovine albumin;

- bottles containing Tween 20 det rgent solution;

- bottles of substrate containing orthophenyl n diamine and hydrogen peroxide in buffer at pH 5.0;

- vials containing solutions of human NGF at different concentrations, including a negative control solution;

- written instructions for the assay.

It is possible to use as a support, for example, tubes, microtitration plates or their strips, beads or disks made of glass, plastic, or other substances suitable for this purpose. Given the type of assay, the antibody does not necessarily have to be bound to a support, and can be present free in solution. Should the antibodies not be bound to a support, it is necessary to utilize a chemical or biological precipitating agent such as polyethylene glycol, or a complexing agent such as protein-A or protein-G.

As labelling/detection agents, it is possible to use enzymes such as horseradish peroxidase, β-galactosidase, or alkaline phosphatase, fluorescent substrates, fluorogenic, chemiluminescent, bioluminescent, radioactive, or metallic molecules, or biotin. If desired, a kit can contain aids for the determination of labelling agents, for example a substrate for the enzyme, and washing or dilution buffers which may or may not contain detergents.

While the present method and kit have been described for the determination of human NGF, and more particularly for the biologically active human form defined as 2.5S or βNGF, the possible applications of anti-peptide antibodies are numerous. For example, such antibodies can be used in immunochemical tests for the quantitative assay of proteins of int rest in biological fluids, and for immunoaffinity chromatography, facilitating th purification of the corresponding antigen, which is often present in very low

concentrations and in very complex biological fluids (Corona G. et al., "Synthetic Peptides: Approaches to biological problems", UCLA Symposia Colorado USA 1980).

Any polypeptide or protein analyte can be qualitatively and/or quantitatively detected by the method of the present invention. Examples of proteins which can be detected by the method of the present invention include, but are not limited to, for example, soluble cytokines and growth factors, including the nerve growth factor of human origin. In order to apply the present method to the detection of other polypeptide or protein analytes, it is first necessary to analyze the hydropathic profile of such analytes to facilitate the selection of potentially useful peptide fragment sequences. Next, antibodies can be raised against such selected peptide fragments. Finally, the extent of cross-reactivity of these antibodies with the entire polypeptide or protein molecule can be determined by routine immunological methods. Those peptide sequences that result in the production of antibodies that effectively cross-react with the entire analyte molecule are useful in the present method and kit, and can be employed in the qualitative and/or quantitative detection of polypeptide or protein analytes as described herein.

As would be apparent to the ordinary skilled artisan, the qualitative and quantitative detection of proteins of medical and veterinary significance via the instant method provides a valuable rapid diagnostic tool permitting the diagnosis and monitoring of a wide variety of diseases and therapies. The latter include, but are not limited to, diseases of the neuroimmunoendocrine type, the neuroimmunodegenerative type, such as Alzheimer's disease, multiple sclerosis, Huntington's disease, motoneuron disease, Guillain Barré syndrome, and Parkinson's disease, diseases of the central nervous system, such as those of traumatic, anoxic, degenerative, or toxic-infective origin, diseases of the peripheral nervous system, such as those of

traumatic-compressive, degenerative, dysmetabolic, or toxic-infective origin, etc.

A further advantage of the present inventive method derives from the fact that the peptide sequence employed to produce the antibodies necessary to carry out the analysis of an analyte-containing fluid can be generated from the nucleotide sequence of the target protein or polypeptide. Knowledge of the amino acid sequence of the protein or polypeptide, or availability of the analyte itself, which may be very expensive or in limited supply, is not required. Furthermore, such antibodies can be obtained by immunization with a peptide without the use of adjuvants. Finally, the length of the peptide fragment required is short, varying between 8-30, and preferably about 17, amino acids.

The invention being thus described, it is clear that the same can be modified in various ways. Such modifications are not to be considered as diverging from the spirit and purpose of the present invention, and any modification which would be apparent to one skilled in the art is considered to come within the scope of the following claims.

CLAIMS

1. A method for the qualitative or quantitative determination of a polypeptide or protein analyte present in a biological fluid or solution, comprising the steps of:

(a) producing antibodies to a peptide fragment of said polypeptide or protein;

(b) reacting a sample of said biological fluid or solution with said antibodies in the presence of said peptide fragment labelled so as to allow detection of complexes of said antibodies with said labelled peptide fragment;

(c) removing any unreacted biological fluid or solution and labelled peptide fragments from the reaction mixture of step (b);

(d) incubating the remaining antibodies with a reagent capable of detecting antibody-labelled peptide fragment complexes; and

(e) qualitatively and quantitatively detecting or measuring said antibody complexes of step (d).

2. The method of claim 1, wherein the amino acid sequence of said polypeptide, said protein, or said peptide fragment is obtained from the nucleotide sequence of said polypeptide or protein.

3. The method of claim 1, wh rein said antibodies are in solution or are bound or adsorbed to a support.

4. Th method of claim 1, wherein said antibody complexes are bound to said support, or are precipitated from solution, prior to detecting or measuring said antibody complexes.

5. The method of claim 1, wherein said antibodies are specific for said analyte.

6. The method of claim 1, wherein said antibodies are obtained by immunization with a peptide present in the amino acid sequence of said analyte.

7. The method of claim 1, wherein said antibodies are obtained by immunization with a peptide present in the amino acid sequence of said analyte without the use of an adjuvant.

8. The method of claim 1, wherein said peptide is present in a hydrophilic portion of said polypeptide or protein analyte.

9. The method of claim 8, wherein said peptide is determined with the aid of a computer program capable of producing the hydropathic profile of said polypeptide or protein analyte.

10. The method of claim 1, wherein said analyte is a soluble cytokine.

11. The method of claim 1, wherein said analyte is a growth factor.

12. The method of claim 1, wherein said analyte is the n rve growth factor of human origin.

13. The method of claim 1, wherein said antibodies are polyclonal antibodies.

14. The method of claim 1, wherein a hapten-peptide capable of being detected via an enzyme reaction is employed as a competition and detection agent.

15. The method of claim 1, wherein said antibodies are specific for human nerve growth factor.

16. The method of claim 15, wherein said antibodies are bound to a solid support.

17. The method of claim 16, wherein said solid support is a microtitration plate.

18. The method of claim 16, wherein said unreacted biological fluid or solution and labelled peptide fragments are removed from said antibodies via the use of a non-ionic detergent.

19. The method of claim 18, wherein said non-ionic detergent is Tween 20.

20. The method of claim 1, wherein said biological fluid or solution is selected from the group consisting of serum, interstitial fluid, lymph, synovial fluid, aqueous humor, urine, cerebrospinal fluid, sweat, tears, saliva, gastric secretions, pancreatic secretion, intestinal secretions, bile, milk, and a culture medium.

21. A kit for the qualitative or quantitative determination of a polypeptide or protein analyte present in a biological fluid or solution, comprising:

anti-analyte antibodies specific for a peptide fragment of said polypeptide or protein analyte which are capable of recognizing said polypeptide or protein analyte; and

a peptide fragment which is capable of being recognized by said antibodies, and which is also capable of being detected by a detection system.

22. The kit of claim 21, wherein said anti-analyte antibodies are prepared by immunization with a peptide fragment, the amino acid sequence of which is within the sequence of said analyte.

23. The kit of claim 22, wherein said peptide fragment is a sequence present in a hydrophilic portion of said analyte.

24. The kit of claim 23, wherein said peptide fragment is determined with the aid of a computer program capable of producing the hydropathic profile of said analyte.

25. The kit of claim 21, wherein said anti-analyte antibodies are prepared by immunization without the use of an adjuvant.

26. The kit of claim 21, wherein said antibodies recognize a peptide portion of human nerve growth factor, as well as human nerve growth factor itself.

27. The kit of claim 26, wherein said antibodies are bound or adsorbed on a solid support.

28. The kit of claim 21, wherein said peptide fragment represents a portion of the amino acid sequence of said polypeptide or protein analyte.

29. The kit of claim 21, wherein said detection system comprises a fluorescent substrate, a coloring agent, a metal, or a radioactive label.

30. The kit of claim 21, wherein said detection system is enzymatic.

31. The kit of claim 30, further comprising an enzyme substrate.

32. The kit of claim 27, wherein said solid support is a polycarbonate microtitration plate.

33. The kit of claim 21, further comprising a standard curve for said polypeptide or protein analyte.

34. The kit of claim 21, further comprising a protein solution containing a detergent.

35. A method for the diagnosis or monitoring of a neuroimmunoendocrine disease in a human or animal, comprising determining the level of a diagnostically relevant polypeptide or protein in a biological fluid of said human or animal according to the method of claim 1.

36. A method for the diagnosis or monitoring of a neuroimmunodegenerative disease in a human or animal, comprising determining the level of a diagnostically relevant polypeptide or protein in a biological fluid of said human or animal according to the method of claim 1.

37. A method for the diagnosis or monitoring of a disease of the central nervous system in a human or animal, comprising determining the level of a diagnostically relevant polypeptide or protein in a biological fluid of said human or animal according to the method of claim 1.

38. A method for the diagnosis or monitoring of a disease of the peripheral nervous system in a human or animal, comprising determining the level of a diagnostically relevant polypeptide or protein in a

biological fluid of said human or animal according to the method of claim 1.

39. A method for the determination of human nerve growth factor in a biological fluid or solution, comprising the steps of:

producing polyclonal antibodies to said human nerve growth factor by immunizing a rabbit with synthetic peptide 20-36 of human nerve growth factor;

adsorbing said polyclonal antibodies onto the surface of the wells of a polycarbonate microtitration plate;

placing samples of said biological fluid or solution in said wells in the presence of peptide 20-36 conjugated to biotin and incubating for 24 hours at room temperature;

removing any excess, unreacted reagent and washing said wells with buffer containing bovine albumin and a detergent;

adding avidin-conjugated enzyme to said wells and incubating for a period of time effective to allow avidin-biotin complexes to form;

removing excess reagent by washing said wells;

adding enzyme substrate to said washed wells in an appropriate buffer and incubating so as to allow formation of the enzymatic reaction end-product;

measuring the amount of said end-product formed; and

relating the amount of said end-product formed to the amount of said human nerve growth factor with the aid of a standard curve for human nerve growth factor.

40. A method for the selection of a peptide fragment sequ nce useful in the method of claim 1, comprising th st ps of:

selecting peptide fragment sequences present in the amino acid sequence of said polypeptide or protein analyte;

raising antibodies against said peptide fragment sequences;

determining the extent of cross-reactivity of said antibodies with the entire molecule of said polypeptide or protein analyte; and

selecting those peptide sequences that result in the production of antibodies that effectively cross-react with said entire analyte molecule for use in the method of claim 1.

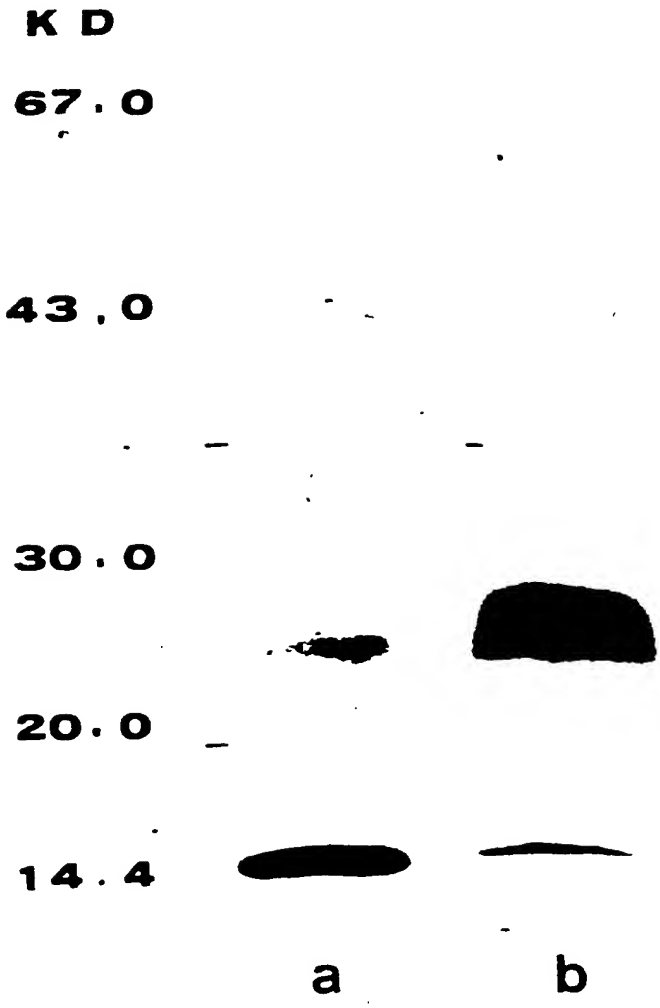

FIG. 1

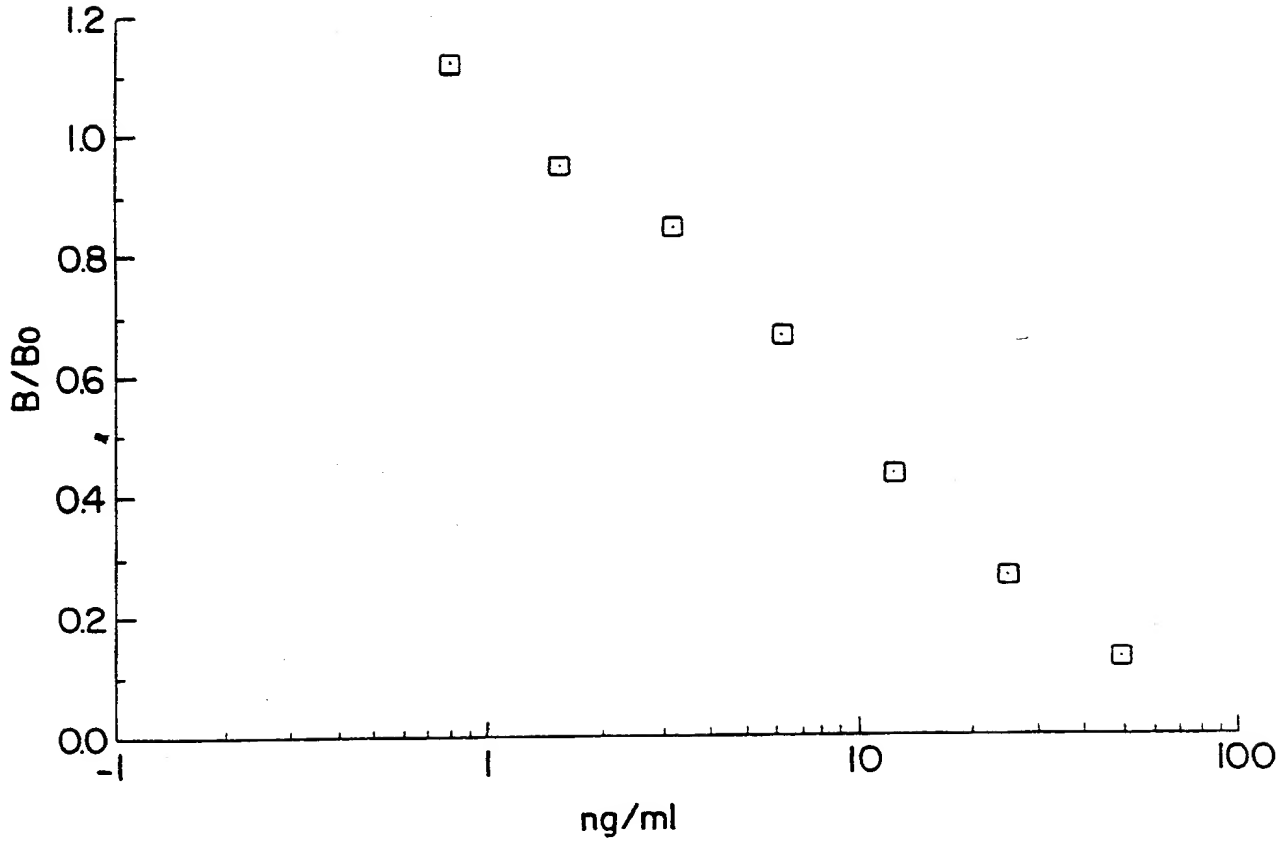

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application PCT/EP 91/01100

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 G 01 N 33/68 G 01 N 33/58 G 01 N 33/543

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	G 01 N C 07 K C 12 P C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP,A,0345906 (MERCK & CO. INC.) 13 December 1989, see page 4, lines 6-20; page 5, line 42 - page 10, line 45; page 11, line 40 - page 13, line 30; page 14, lines 14-45; page 22, lines 37-57	1-6,13, 14,20-22,28-30,33
Y		10-12, 15-17, 23,26, 27,32, 35-40

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23-09-1991	11 OCT 1991
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	[illegible]

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Journal of Cellular Biochemistry, Supplement 14C, UCLA Symposium on Molecular & Cellular Biology, 3 February - 11 March 1990, abstract CK302, G. Corona et al.: "Properties of anti-peptide antibodies raised against the synthetic peptide 20-36 of nerve growth factor", page 53, see the abstract	10-12, 15-17, 23,26, 27,32, 35-40
X	WO,A,8903430 (TERRAPIN DIAGNOSTICS, LTD) 20 April 1989, see page 6, lines 5-10; page 11, line 10 - page 12, line 18; page 39, line 22 - page 4, line 9	1-6,13, 14,20-22,28-30,33
Y		8,10-12 ,15-17, 23,26, 27,32, 35-40
Y,P	WO,A,9010644 (LOPE MEDICINE AB) 20 September 1990, see pages 1-5; page 11, lines 10-15	8,10-12 ,15-17, 23,26, 27,32, 35-40
A	D.T. Krieger et al.: "Brain Peptides", 1983, chapter 20, O.P. Rorstad: "Competitive binding assays", pages 465-483, see pages 466-473	1,3-6, 10-14, 20
A,P	EP,A,0418590 (TAKEDA CHEMICAL INDUSTRIES LTD) 27 March 1991, see pages 1-3; page 6, lines 9-45; page 8, lines 1-38; page 9, lines 11-53; page 21, lines 20-51	1-40

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101100 SA 48215

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0345906	13-12-89	JP-A- 2117699	02-05-90
WO-A- 8903430	20-04-89	AU-A- 2790989	02-05-89
		EP-A- 0387276	19-09-90
WO-A- 9010644	20-09-90	AU-A- 5339390	09-10-90
		SE-A- 8900899	15-09-90
EP-A- 0418590	27-03-91	None	